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(54) Title: FACTOR IX - POLYMERIC CONJUGATES (57) Abstract Conjugates containing a substance with coagulant activity, such as recombinant Factor IX, non-antigenic polymers, such as poly(ethylene glycol), are disclosed. Also disclosed are methods of forming the novel conjugates of this invention.		

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FACTOR IX - POLYMERIC CONJUGATES

The present invention is directed to methods of modifying substances having Factor IX activity, such as the pro-coagulant glycoprotein Factor IX and, more particularly, to methods for the modification of such substances with non-antigenic polymers, such as polyethylene glycol, and the resultant conjugates.

Background of the Invention

The process of blood clotting typically begins with trauma to a blood vessel. The damaged vessel wall initiates hemostasis by causing adherence and accumulation of platelets at the injury site and by activating plasma proteins which initiate the coagulation process. A series of proteins, including Factor IX, are activated sequentially by specific proteolytic cleavages and conformational changes leading to the deposition of insoluble fibrin which curtails the blood flow.

Blood coagulation Factor IX circulates as an inactive zymogen. It enters the coagulation cascade after activation in the presence of Factor XIa or VIIa and tissue factor. The activated Factor IX (IXa) in turn activates Factor X in the presence of Factor VIIIa, phospholipids and calcium.

Hemophilia is a bleeding disorder caused by the lack of an essential blood factor. Hemophilia A (classic hemophilia) is the most common and is caused by a genetic deficiency or abnormality of Factor VIII. Hemophilia B is caused by a deficiency or abnormality of Factor IX. The genetic deficiency of Factor IX results in hemophilia B. It has been estimated that about 3000 patients in the U.S. alone suffer from hemophilia B.

Hemophilia is a heterogeneous disorder. Mutant blood Factor IX proteins can have clotting activities that vary from near normal to severely deficient. The clinical features of hemophilia A and B are identical. Less than 1% clotting factor activity is defined as severe hemophilia, and is accompanied by spontaneous bleeding in the muscles and larger joints. Repeated bleeding in joints causes arthropathy or hemarthroses which is a major chronic complication. Hemarthroses are worsened by muscle atrophy due to muscle bleedings. Often, repeated hemarthroses result in eventual deformity and crippling. In mild hemophilia (5-40% FVIII or FIX activity), bleeding does not occur except after trauma. Moderately severe hemophilia (1-5% FVIII or FIX activity) has clinical features between the severe and mild hemophilia.

Conventional treatment of hemophilia B consists of replacement of the deficient Factor IX from pooled donor plasma including fresh, frozen plasma or Factor IX concentrates. Pooled donor preparations, however, have been associated with the transmission of viral diseases such as hepatitis (eg. B, delta, non A non B) and HIV. This is in spite of increased purification techniques to reduce the virus load. It has been estimated that 50% of hemophilia patients are either hepatitis positive or HIV positive. These viral infections are now the major cause of morbidity and mortality in patients with hemophilia.

Further disadvantages of pooled donor plasma include the cost and availability of the purified blood factors. With increased purification steps, the cost of blood factor therapy has increased. Availability of the blood factors is also a concern. Theoretically, these

factors should be administered prophylactically in many cases to avoid the sequelae of uncontrolled bleeding such as the development of joint disorders. However, cost, availability and the pharmacokinetics make an effective prophylactic therapy unfeasible.

With the advent of DNA technology, researchers have now cloned and are testing a number of recombinant blood factors, including Factor IX in patients with hemophilia. While recombinant technology may overcome the problems of viral contamination and availability, it does not affect the pharmacokinetics of the factors nor the formation of inhibitors (antibodies) in patients. It is estimated that 2-3% of all patients with hemophilia B will develop IgG antibodies that will nullify the value of replacement therapy. Inhibitor development occurs primarily in patients with severe hemophilia although antibodies to Factor IX in mild hemophilia have been reported. Approximately 5-15% of patients with severe hemophilia have antibodies to Factor VIII or IX. It has been estimated that the actual risk of developing neutralizing antibodies by age 20 is as high as 15-24%. Joint bleedings often cannot be controlled and adequately treated and many of these patients are severely handicapped.

To overcome the neutralizing effect of the antibodies, physicians can be forced to increase the dosage to the factor. However, there is often a decreased response to the replacement therapy despite increases in dosage. Care in administering the factors as well as the administration of steroids and other immunosuppressive agents such as azathioprine, cyclophosphamide and high-dose gammaglobulin G is often

required to prevent or limit the development of antibodies and hypersensitivity reactions. Antibody depletion through plasmapheresis has been used to decrease the inhibitor titers in circulation. However, such immuno-suppressive techniques have been only partially successful and raise the risk that the patient will be more vulnerable to opportunistic infections, e.g. HIV or hepatitis.

In light of the complications and risks inherent in the conventional treatment of hemophilia B, it is desirable to provide compositions having Factor IX activity which are less likely to cause the formation of inhibitor antibodies. In light of the high costs of Factor IX, it is also highly desirable to increase the in vivo circulating life of Factor IX activity.

Summary of the Invention

The various embodiments of the present invention provide conjugates having Factor IX activity with reduced propensities to develop problems relating to immunogenecity and antigenicity. In addition, there are also provided methods for the modification of substances such as glycopolypeptides or fractions thereof having Factor IX activity with non-antigenic polymeric materials, such as polyethylene glycol (PEG). The Factor IX substance included in the conjugates is preferably one which has been formed using recombinant technology. The Factor IX substance may also be derived from human or animal plasma sources, such as bovine or porcine plasma. Transgenic sources are also contemplated. As used herein, the term "Factor IX substance or fraction" means any substance which demonstrates the ability in vivo to

function as mammalian Factor IX, i.e. activate Factor X and continue the intrinsic clotting cascade. The Factor IX fraction can also comprise other proteins and reagents, as well as other serum proteins including albumin, fibrin, fibrinogen, etc., which do not interfere with in vivo activity.

An advantage of one preferred embodiment of the present invention lies in the fact that the substantially non-antigenic material is attached to the activation region of the Factor IX. Since Factor IX is activated by the proteolytic removal of the activation region, the substantially non-antigenic polymer is removed from the Factor IX upon activation and therefore does not interfere with the normal functioning of the activated protein. Thus the benefits normally achieved with conjugation of a non-antigenic polymer can be obtained producing a long-circulating procoagulant without interfering with the structure or physiologic activity of the activated Factor IX.

According to one embodiment of the present invention, substances containing Factor IX activity are reacted with a substance capable of oxidizing vicinal diols of carbohydrate moieties in the activation region to form aldehydes. One or more substantially non-antigenic polymers can then be covalently attached to the oxidized carbohydrate moieties. The polymers are preferably activated or functionalized to include a terminal hydrazine group such as, for example, an acyl hydrazine. These reactions are performed under conditions sufficient to effect conjugation while maintaining at least a portion of the Factor IX activity. The resulting Factor IX may then be stabilized in a

manner known to those of ordinary skill in the art with human serum albumin (HSA) or glycine, sterile filtered and, if desired, lyophilized. This method has been found to provide a long-circulating Factor IX-acting conjugate which is less susceptible to antibody inhibitors and retains an effective amount of the unmodified substance's original activity.

These and other embodiments of the present invention are described in detail below.

Brief Description of the Drawings

Figure 1 is a schematic representation of Factor IX showing the amino acid sequence, structure and various domains.

Detailed Description

The various embodiments of the present invention advantageously provide methods for modifying substances having Factor IX activity with substantially non-antigenic polymeric substances under relatively mild conditions, for example at a pH of about 5-7 with about 5.5 - 6.5 preferable and at temperatures which do not exceed 37°C, and are preferably in the range of from 10-25°C. Those skilled in the art will appreciate, for example, that human Factor IX is a relatively large and sensitive glycoprotein. The present invention provides methods for modifying such glycoproteins without subjecting them to conditions which would eliminate their activity.

The substantially non-antigenic polymer substances included in the conjugates are preferably poly(alkylene oxides). Within this group of substances are alpha-substituted polyalkylene oxide derivatives such as methoxypolyethylene glycols or other suitable alkyl-substituted derivatives such as those containing C₁-C₄ alkyl groups. It is preferred, however, that the non-antigenic material be a monomethyl-substituted PEG homopolymer. Alternative polymers such as other polyethylene glycol homopolymers, polypropylene glycol homopolymers, other alkyl-polyethylene oxides, bis-polyethylene oxides and co-polymers or block co-polymers of poly(alkylene oxides) are also useful. In those aspects of the invention where PEG-based polymers are used, it is preferred that they have molecular weights of from about 200 to about 10,000. Molecular weights of about 2,000 to 7,000 are preferred and molecular weights of about 5,000 are particularly preferred. Alternatively, other non-antigenic polymers such as dextran, polyvinyl pyrrolidone, starches, complex carbohydrates and other substantially non-antigenic compounds known in the art may also be linked to a compound exhibiting Factor IX activity in vivo by covalent or non-covalent modification.

Human Factor IX is a single chain glycoprotein of 57,000 Daltons. Structurally, it can be considered as having four essential domains (Figure 1). The Gla or gamma carboxy-glutamate rich domain, the EGF-like regions, the activation peptide and the active site. The Gla domain encompasses the first 46 residues including 12 gamma carboxy-glutamate residues. Residues 47-127 form the EGF-like region. The activation region encompasses residues 128-195 and the two cleavage sites for Factor

XIa are Arg144-Ala146 and Arg180-Val181. The activation peptide, which is unbound upon activation, contains 35 amino acid residues and two Asp-linked carbohydrate side chains. The final 220 residues form the heavy chain of activate Factor IX and contain the enzyme active site.

As stated above, one particularly preferred embodiment includes recombinantly prepared substances having Factor IX activity in vivo. A non-limiting list of suitable recombinant glycoproteins include those described in J. Biological Chemistry, Vol. 261, No. 211, pp. 9622-9628, (1986); J. Biological Chemistry, Vol. 267, No. 21, pp. 14759-14766 (1992); and Nature, Vol. 316, pp. 271-273 (1985). The contents of each of the foregoing are incorporated by reference herein.

It will be understood that in those situations where recombinant sources of Factor IX activity substances are selected, modifications such as amino acid substitutions or other mutants which do not effect or eliminate Factor IX activity are within the scope of the present invention. Moreover, in those situations where non-human Factor IX substances are included, some differences in homology are recognized. These alternatives, however, have the desired in vivo activity and the preferred activation region polymeric attachment site(s).

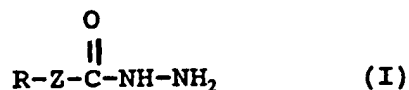
As stated above, covalent modification of the protein material is preferred. The modification reaction includes reacting a substance having the desired Factor IX activity with a substantially non-antigenic polymeric substance under conditions sufficient to effect conjugation while maintaining at least a portion of the

Factor IX activity.

Preferably, one or more polymer chains are covalently attached to the Factor IX by reacting suitably activated non-antigenic polymers with oxidized carbohydrate moieties in the activation region. Reactive carbonyl groups can be generated on saccharide units of the carbohydrate moieties, for example, by oxidizing vicinal diols with excess sodium periodate utilizing steps understood by those of ordinary skill in the art. The reactive carbonyls can also be generated enzymatically, e.g., by use of galactose oxidase or neuraminidase and galactose oxidase. For purposes of the present invention, enzymatic oxidation is preferred.

"Suitably activated non-antigenic polymers" is understood by those of ordinary skill in the art to mean that the polymer is functionalizing to include a group capable of linking the polymer to the Factor IX substance. Preferably, the functionalized moiety is reactive with oxidized carbohydrate moieties, such as hydrazine.

Preferred hydrazine activated polymers of the present invention are acyl hydrazines having a structure corresponding to Formula I:



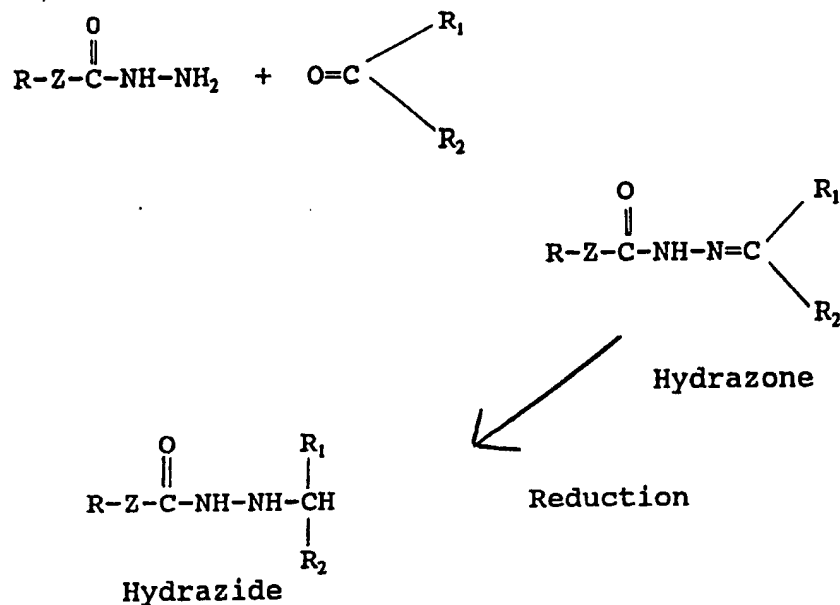
wherein R is one of the above-disclosed water-soluble non-antigenic polymers and Z is O, NH, S or a lower alkyl group containing up to ten carbon atoms. See, for example, commonly assigned PCT Int'l. Publication No. WO92/16555 which is incorporated by reference herein.

The acyl hydrazine derivative in which Z is O (mPEG-Carbazate) is prepared by reacting, for example, the methoxypolyethylene glycol-N-succinimide carbonate described in U.S. Patent No. 5,122,614 with an excess of hydrazine in an organic solvent in which the reactants are soluble, such as methanol, methylene chloride, or toluene. The disclosure of U.S. Patent No. 5,122,614 with respect to the preparation of methoxypolyethylene glycol-N-succinimide carbonate is hereby incorporated by reference. After the reaction is completed, the solvents and excess hydrazine are then removed utilizing conventional techniques.

The preparation of acyl hydrazine polymer derivatives is described with reference to MPEG for purposes of illustration, not limitation. Similar products would be obtained with any of the polymers suitable for use with the present invention, and it will be clear to those of ordinary skill in the art how this preparation can be adapted to the other suitable polymers.

The reaction of the acyl hydrazines of Formula I with a glycoprotein having Factor IX activity to form a hydrazone linkage is illustrated by the reaction sequence of Scheme 1. R and Z are the same as described above and R₁ and R₂ form an oxidized carbohydrate moiety on which reactive carbonyl groups have been generated:

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Scheme 1

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In one aspect of this embodiment, the hydrazone can be reduced to the more stable alkyl hydrazide by reacting the hydrazone with, for example, NaBH_4 or NaCNBH_3 . Alternatively, the hydrazone linkage is maintained to provide a prodrug-like conjugate. In this

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embodiment, the aqueous plasma environment allows hydrolysis of the hydrazone bond, regenerating the starting glycoproteins in vivo.

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The process of the present invention includes preparing or providing the activated polymer and thereafter reacting it with a Factor IX protein having at least one oxidized carbohydrate moiety. The carbohydrate moieties can be oxidized by reacting the glycoprotein in aqueous solution with sodium periodate or enzymatically

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using galactose oxidase or combination of neuraminidase and galactose oxidase as disclosed by Solomon et al., J. Chromatography, 510, 321-9 (1990). The reaction is

preferably run at temperatures of about 0-10 C° when reacting with sodium periodate or at about room temperature when the carbohydrate moieties are being oxidized enzymatically. The reaction medium is preferably buffered, depending upon the requirements of the glycoprotein. The oxidized glycoprotein is then recovered and separated from the excess periodate by column chromatography, dialysis, ultrafiltration or the like.

The reaction is carried out in a buffer such as 0.1 M phosphate buffer at a pH of from about 5.0 to about 8.0. The F.IX having at least one oxidized carbohydrate moiety is reacted with an appropriate amount of the activated polymer, which is typically present in a molar excess relative to the F.IX. The polymeric excess preferably ranges from about 10 to about 100 molar ratio excess of the polymer of the F.IX. The reaction is carried out at temperatures from about 0 to about 37°C over time periods ranging from a few minutes to as long as 24 hours. Temperatures from about 10 to about 25°C are preferred and time periods of about 2 hours are sufficient to carry out the conjugation reaction.

In addition to or as an alternative to the above-described carbohydrate-directed modification, further polymeric modification is possible. For example, epsilon amino lysine groups can be modified with appropriately functionalized polymers or additional active moieties. See, for example, U.S. Patent No. 4,179,337 and 5,122,614, which are incorporated by reference herein.

Following the conjugation reaction, the desired

product is recovered using known techniques and purified using column chromatography or similar apparatus, if necessary. Depending upon the reaction conditions, the conjugates have from about 1 to about 10 polymeric chains attached to the glycoprotein. By controlling the molar excess of the oxidant and the polymer with respect to the glycoprotein, the artisan can tailor the number of polymeric chains attached. Conjugates containing from about 3 to about 8 polymeric chains are preferred, while conjugates containing from about 3 to about 5 polymeric chains are most preferred.

Another aspect of the present invention provides methods of treating hemophilia B. For purposes of the present invention, treating includes prevention and/or prophylaxis. The methods include administering, in a pharmaceutically acceptable vehicle, such as a parenteral solution, an effective amount of the compositions described herein to alleviate clotting deficiencies. Those of ordinary skill in the art will realize that the amount of the conjugate used in the method of the present invention will vary somewhat from patient to patient, however, conjugates capable of delivering from about 0.1 IU/kg to about 10 IU/kg per administration or an amount sufficient to achieve or maintain a level of between .2 IU/ml - 5 IU/ml blood are preferred. The optimal dosing of the conjugate can be determined from clinical experience. Essentially, the objective of treatment is to normalize serum F.IX levels and provide normal clotting times for Hemophilia B patients.

Modification of Factor IX with Polyethylene Glycol (PEG)Example 1

Purified human pooled Factor IX (3.7 mg/ml) obtained from the American Red Cross was dialyzed overnight against 50 mM sodium phosphate pH 6.25, 100 mM NaCl. To 2.5 mg. of Factor IX (4.4×10^{-5} mmol) was added a 300 fold molar excess of NaIO_4 (69 μl of a 40 mg/ml solution) to oxidize the carbohydrate moieties. The reaction mixture which was left to incubate on ice for one hour in the dark. A 200 fold molar excess of PEG-carbazate was then added and the reaction allowed to proceed overnight at 4°C. The resulting solution was then dialyzed against 50mM sodium phosphate pH 7.0, 100 mM NaCl using a Pierce microdialyzer system. The activity of the PEG modified Factor IX, oxidized Factor IX and an unmodified control were determined in the presence of FIX- deficient plasma using a Dade chromogenic assay kit, available from Baxter, Inc., Deerfield, IL and are set forth below.

<u>SAMPLE</u>	<u>SPECIFIC ACTIVITY (U/MG)</u>
FIX Control	134
Oxidized FIX	120
PEG-FIX	111

EXAMPLE 2

Purified Factor IX (3.7 mg/ml) obtained from the American Red Cross was desalted over a PD-10 column (Pharmacia) into 50 mM sodium citrate pH 5.5, 150 mM NaCl. Carbohydrate residues were oxidized by the addition of NaIO_4 to 5 mM and incubated on ice for 20 min. in the dark. Sucrose was added to 10mM to quench

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the reaction and the entire mixture was desalted as described above. The oxidized Factor IX was then incubated with a 100 fold excess of PEG-hydrazide for 2 hr. at room temperature. The resulting hydrazone was
5 chilled on ice for 10 min. then reduced to the corresponding hydrazide by treating with 5 mM NaCNBH₄ at 4°C overnight. The PEG-Factor IX was purified by GPC-HPLC using a Showdex KB804 column eluted with 50 mM histidine pH 6.5, 5mM CaCl₂, 100 mM NaCl. The purified
10 sample was concentrated and aliquots were stored at either 4°C, -70°C or lyophilized. The specific activities were determined in the presence of Factor IX deficient plasma using a Dade Factor IX chromogenic assay kit available from Baxter, Inc., Deerfield, IL and in a
15 one stage APTT clotting assay.

	<u>SAMPLE</u>	<u>SPECIFIC ACTIVITY (U/mg)</u>	
		<u>Chromogenic</u>	<u>Clotting</u>
20	FIX Control	93	197
	PEG-F.IX (4°C)	123	16
	PEG-F.IX (-70°C)	168	10
25	PEG-F.IX (Lyoph)	206	51

EXAMPLE 3

30 American Red Cross Factor IX (18.5 mg, 3.7 mg/ml) was desalted over a 30 ml Sephadex G-25M column into 50 mM citrate pH 5.5, 150 mM NaCl. The protein was treated with 5mM NaIO₄ for 20 min at 4°C in the dark. The reaction was quenched by the addition of sucrose to
35 10mM and the mixture was desalted as described above. A 100-fold molar excess of PEG-Carbazate was added to the oxidized protein and incubated at room temperature for 2

hr. After cooling on ice for 10 min. NaCNBH_4 was added to 5mM and the reaction mixture was incubated on ice for 2 hr. The PEG-Factor IX was purified by GPC-HPLC using a Showdex KB804 column eluted with 50mM histidine pH 6.5, 5mM CaCl_2 , 100mM NaCl. Activities were determined in the presence of Factor IX deficient plasma using a Dade chromogenic assay kit and in a one stage APTT clotting assay.

10	<u>SAMPLE</u>	<u>SPECIFIC ACTIVITY (U/mg)</u>	
		<u>Chromogenic</u>	<u>Clotting</u>
	F.IX control	106	288
15	PEG-F.IX	45	69

EXAMPLE 4

20 1 ml of purified Factor IX (4.75 mg/ml) obtained from Alpha Therapeutics, Los Angeles, CA (Lot# 52018), was desalted on a PD-10 column equilibrated with 100 mM NaAc pH 5.5, 150 mM NaCl. Fractions containing protein were pooled and 0.5 M NaIO_4 was then added to a final concentration of 5mM. The oxidation proceeded on ice for 20 minutes. The reaction was quenched by the addition of 0.1 M sucrose to a final concentration of 10 mM and was allowed to sit on ice for 5 minutes. Excess periodate and sucrose were removed by desalting on a PD-10 column as described above. A 100 fold excess PEG-Hydrazide was added, and the reaction proceeded for 2 hours at room temperature. After cooling on ice, 0.5 M NaCNBH_4 was added to a final concentration of 5 mM and the mixture was kept refrigerated overnight. Excess PEG and NaCNBH_4 were removed by GPC-HPLC using a Showdex column equilibrated with 0.1 M sodium phosphate pH 7.5.

SDS-PAGE of the purified material revealed a molecular weight shift to around 150-225 kD with no trace of native F.IX.

5 The sample was concentrated to approximately 1
ml using a centricon 30 centrifugal concentrator. Protein determination was performed using a Pierce BCA protein assay kit and Factor IX activity was determined using a Dade Factor IX chromogenic assay kit in the
10 presence of Factor IX deficient plasma. The PEG-F.IX was calculated to retain 85% activity.

EXAMPLE 5

15 Purified Factor IX (49.4 mg) obtained for Alpha Therapeutics Corporation, Los Angeles, CA was buffer exchanged using a 30K NMWL cutoff miniultrasette tangential flow apparatus (Filtron) into 50 mM citrate,
20 pH 5.5 150 mM NaCl. The volume was adjusted to approximately 30 ml and the protein was treated with 5 mM NaIO₄ for 20 min., on ice, in the dark. The oxidation reaction was quenched with 10 mM sucrose and the mixture was buffer exchanged as described above. A 100-fold
25 molar excess of PEG-carbazate was added and the coupling reaction was incubated at room temperature for 2 hr. After cooling on ice for 10 min., NaCNBH₃ was added to 5 mM and the reaction mixture was incubated at 4°C overnight. The reaction mixture was desalted using a
30 miniultrasette apparatus with 50 mM histidine, pH 6.2, 5 mM CaCl₂, 100 mM NaCl with 10 mg/ml glycine. The sample was aliquoted and stored at either 4°C, -70°C or lyophilized. SDS-PAGE revealed an increased and broad molecular weight distribution but no sign of

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contaminating native protein. Specific activities were *determined in the presence of Factor IX deficient plasma using a Dade chromogenic assay kit.

5	<u>SAMPLE</u>	<u>SPECIFIC ACTIVITY (U/mg)</u>
	Native Factor IX	47
	PEG-F.IX (4°C)	128
	PEG-F.IX (-70°C)	146
10	PEG-F.IX (Lyoph)	115

15 The various embodiments of the present invention, therefore, provide conjugates which retain significant levels of Factor IX activity while having less of a tendency to cause the formation of inhibitor antibodies.

WE CLAIM:

1. A biologically active conjugate comprising a first substance having Factor IX activity bound to a substantially non-antigenic polymeric substance.
2. The conjugate of claim 1 wherein said first substance is bound to said substantially non-antigenic polymeric substance by a linkage containing a hydrazide or hydrazone functional group.
3. The conjugate of claim 1 wherein said first substance comprises Factor IX.
4. The conjugate of claim 3 wherein said Factor IX is of recombinant origin.
5. The conjugate of claim 3 wherein said Factor IX is of mammalian origin.
6. The conjugate of claim 5 wherein said origin of said mammalian F.IX is selected from the group consisting of human, porcine, bovine, and mixtures thereof.
7. The conjugate of claim 3 wherein said Factor IX is of transgenic origin.
8. The conjugate of claim 1 wherein said first substance comprises fractions of the protein Factor IX.
9. The conjugate of claim 1 wherein said polymeric substance comprises a poly(alkylene oxide).

10. The conjugate of claim 9 wherein said polymeric substance comprises an alpha-substituted polyalkylene oxide derivative.

11. The conjugate of claim 9 wherein said polymeric substance is selected from the group consisting of polyethylene glycol homopolymers, polypropylene glycol homopolymers, alkyl-capped polyethylene oxides, bis-polyethylene oxides and copolymers or block copolymers of poly(alkylene oxides).

12. The conjugate of claim 9 wherein said polymeric substance comprises poly(ethylene glycol).

13. The conjugate of claim 9 wherein said polymeric substance has a molecular weight of about 1,000 to about 17,000.

14. The conjugate of claim 13 wherein said polymeric substance has a molecular weight of about 2,000 to about 7,500.

15. The conjugate of claim 13 wherein said polymeric substance has a molecular weight of about 5,000.

16. The conjugate of claim 1 wherein said substantially non-antigenic polymeric substance is selected from the group consisting of polyvinyl pyrrolidone, dextran, complex carbohydrates, starches and mixtures thereof.

17. A method of preparing a conjugate having Factor IX activity comprising reacting a first substance having Factor IX activity with a substantially non-antigenic polymeric substance under conditions sufficient to effect conjugation of said first substance and said polymeric substance with a linkage containing a hydrazide or hydrazone functional group while maintaining at least a portion of the activity of the first substance.

18. The method of claim 17 wherein said polymer is a poly(alkylene oxide).

19. The method of claim 17 wherein said polyalkylene oxide is an alpha-substituted polyalkylene oxide derivative.

20. The method of claim 19 wherein said poly(alkylene oxide) is a polyethylene glycol.

21. The method of claim 17 wherein said reacting step comprises providing a molar excess of said substantially non-antigenic polymeric substance relative to said first substance.

22. The method of claim 21 wherein said reacting step comprises providing about a 10-20 fold molar excess of said substantially non-antigenic polymeric substance relative to said first substance.

23. The method of claim 17 wherein said reacting step is conducted at temperatures of up to about 27°C.

24. The method of claim 23 wherein said reacting step is conducted at temperatures of about 2 - 10°C.

25. The method of claim 17 wherein said first substance comprises Factor IX.

26. A method of treating hemophilia B comprising administering a therapeutically effective amount of the conjugate of claim 9.

27. A method of treating hemophilia B comprising administering a therapeutically effective amount of the conjugate of claim 1.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/06388**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : C08H 1/00; C08L 89/00; C12N 9/00; A61K 37/00

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/381, 815, 829, 810, 812, 813, 816; 435/219; 525/54.1, 54.2, 54.24; 514/8, 21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US, A, 4,970,300 (FULTON et al.) 13 November 1990. See column 4, lines 21-45 for binding factor VIII to dextran to decrease immunogenicity. See column 16, lines 13-49 for treatment of hemophilia.	1, 3-16, 26, 27
Y	BUSBY et al. "Expression of Active Human Factor IX in Transfected Cells", Nature, Vol. 316, July 1985 pages 271-273. Note the Abstract for production of human Factor IX by use of recombinant techniques.	1, 3-16, 26, 27

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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INTERNATIONAL SEARCH REPORT

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